Nutritionally enhanced plants.

Field of the invention

The present invention relates to the field of improving nutritional content in plants. More particularly the invention relates to the modification of selected plants to improve their content of oestrogenic compounds and to the plants and plant derived products obtainable therefrom.

Background to the invention

Isoflavones are a group of oestrogenic compounds which belong to the flavonoid class of plant secondary metabolites. These compounds are produced naturally in certain plants expressing the enzyme isoflavone synthase and in particular in leguminous plants. The presence of isoflavones is known to provide several advantages including the facilitation of antimicrobial plant defences and establishing bacterial or fungal symbioses within plants as well aiding nitrogen fixation in root nodules.

In addition to the advantages that are conferred to plants, the dietary presence of isoflavones is also believed to provide benefits to human health. For example, dietary isoflavones are believed to be effective at reducing the risk of cancer and cardiovascular disease.

At present, in the human diet the only sources of isoflavones are certain legumes, such as soybean or chickpea. Soy constitutes by far the major dietary source, however supplementation of food products with soy or soy-extracts may adversely affect the flavour profile. It would therefore be desirable to extend the range of plants or plant tissues capable of providing an effective source of isoflavones to the human diet and in particular a source which does not adversely affect the flavour profile of a product.

WO 00/53771 teaches that to form the isoflavone daidzein in transgenic plants that do not possess an isoflavonoid pathway and thus do not produce isoflavones in nature, it would be necessary to introduce therein three new genes, namely chalcone reductase (CHR) to co-act with chalcone synthase (CHS) to form 2',4',4'-trihydroxychalcone, a suitable chalcone isomerase (CHI) to convert 2',4',4'-trihydroxychalcone to liquiritigenin, and isoflavone synthase (IFS).

The applicants have now found that the approach disclosed in WO 00/53771 does not allow the formation of daidzein in respect of many plants. Furthermore the applicants believe that the transformation of the tomato plant as exemplified in example 3 of WO 00/53771 is most unlikely to produce tomatoes with increased levels of daidzein as purported to be achieved therein.

Studies carried out by Yu et al., (Production of isoflavones genistein and daidzein in non-legume dicot and monocot tissues. Plant Physiol. 2000 124:781-793) applied transcription factors C1 and R with the co-introduction of CHR and IFS into non-differentiated Black Mexican Sweet (BMS) maize cultures. This approach yielded only trace elements of daidzein in a single cell line.

The use of this single cell system in drawing any conclusions relating to enzymology and regulation of secondary metabolic pathways in differentiated tissues is recognised in the art as unreliable (Stafford H.A.(1990) CRC Press, Boca Raton, Florida p. 225-239).

BMS maize cell cultures are undifferentiated and are not active in flavonol biosynthesis.

The objective technical problem to be solved by the present invention therefore relates to the need to provide novel plants

which comprise significantly increased levels of daidzein and/or daidzein derivatives.

It has now been found that the solution to this problem lies in a process which selects a non-isoflavone producing plant or part thereof comprising both active anthocyanin and flavonol pathways and alters said plant to increase the enzyme activity of chalcone reductase and isoflavone synthase therein.

At the time of filing it was not known that the selection of a non-isoflavone producing plant comprising both active anthocyanin and flavonol biosynthetic pathways in combination with an increase in these specific enzyme activities could be used to provide plants with increased levels of daidzein and/or derivatives thereof.

Definition of terms

A non-isoflavone producing plant is suitably defined by the absense of isoflavone synthase enzyme activity which renders the tissues of the plant unable to produce isoflavones. The absence of isoflavone synthase activity can be determined by achieving a negative result in a standard enzyme assay as disclosed in Jung et al., (Nature Biotech. Vol. 18 Feb 2000 p. 208-212) incorporated herein by reference.

The term 'plant or part thereof' is used herein to refer to an entire plant or differentiated group of cells forming a part thereof. A part of a plant for the purpose of the invention may relate to leaves, stems, fruit, seeds, flowers, roots, tubers.

The expression 'increasing' is used in comparison to an equivalent unmodified plant or part thereof and may be on an absolute dry weight basis or in relative terms. Except for the modifications introduced by the process of the invention, this equivalent plant is genetically identical thereto.

Daidzein as used herein is taken to comprise 7,4'-dihydroxyisoflavone. Derivatives of daidzein are taken to comprise those molecules which result from the cellular biochemical modification of daidzein. Preferably a daidzein derivative is selected from the group comprising pterocarpans e.g.medicarpin, glyceollin, isoflavanones e.g.vestitone, rotenoids e.g. munduserone, isoflavans e.g. vestitol, α-methyldeoxybenzoins e.g. angolensin, 2-arylbenzofurans e.g. centrolobofuran, isoflavonols e.g. 7,2'-dihydroxy-4'-methoxyisoflavonol, isoflav-3-enes e.g. haginin, 3-arylcoumarins e.g. glysyrin, coumestans e.g. coumestrol, coumaronochromones e.g. lupinalbin, coumaranochromene e.g.pachyrrhisomene.

Derivatives of daidzein may also result from one or more chemical processes selected from the group comprising methylation, glycosylation, prenylation and ether linkage.

A plant or part thereof that is active in anthocyanin biosynthesis has an active anthocyanin pathway and is taken to comprise a tissue which comprises mRNA encoding one or more enzymes selected from the group comprising dihydroflavonol reductase, proanthocyanidin synthase, and UDP-glucose:flavonoid-3-O-glucosyltransferase.

For the purpose of the present invention active anthocyanin biosynthesis may be determined in a plant tissue by a spectrophotometric test wherein absorbance of a hydrolysed plant extract at λvis-max 480-560nm is indicative of an active anthocyanin pathway. Plant tissues are ground in liquid nitrogen and extracted with 80% (v/v) ethanol at 100mg/700µl for 30min at room temperature (~22/C). Following extraction the cell debris is removed by filtration through a 0.45µm Millex_HV filter unit (Millipore Corp, USA). The ethanol extract (360µl) is mixed with 12M HCl (40µl). The acidified ethanol extract is assayed by

spectrophotometer and absorbance determined as $A_{\lambda vis\text{-}max\ 480\text{-}560nm}$, with the A_{657} subtracted.

It is preferred that a plant or the part thereof that is active in anthocyanin biosynthesis contains more than 10mg/kg fresh weight anthocyanin, more preferably at least 100mg/kg, further preferred at least 1000mg/kg and most preferred from 1000 to 10,000mg/kg fresh weight. Suitably this is calculated from absorption values according the formula $C = A*MW*10^3*DF/(\varepsilon*1)$ in which C refers to concentration, A refers to absorption (as defined above); MW is molecular weight; DF is dilution factor; ε is molar extinction coefficient (29,600 for cyanidin 3-glucoside the major anthocyanin in nature) and l is the path length.

A plant or part thereof which is active in flavonol biosynthesis has an active flavonol pathway and is taken to comprise any tissue which comprises mRNA encoding one or more enzymes selected from the group comprising chalcone synthase, chalcone isomerase, flavanone 3-hydroxylase, flavonol synthase.

For the purpose of the present invention whether a plant is active in flavonol biosynthesis may be determined by preparing a hydrolysed tissue extract and detection by HPLC analysis.

For extraction, tissues are harvested and flash frozen in liquid nitrogen before being stored at -80°C. The tissues are then ground to a fine powder to ensure a homogeneous mix. An aliquot from this mixture is then extracted for 30min at room temperature (~22°C) in 80% (v/v) ethanol at 100mg/700µl. Following extraction, the cell debris is removed by filtration through a 0.45µm Millex-HV filter unit (Millipore Corp, USA). The filtrate is stored at -20°C prior to HPLC analyses.

For hydrolysed extracts, 40µl of 12M HCl is added to 360 µl from each tissue extract, before incubating at 90°C for 40 min.

After hydrolysis, an aliquot from each extract is filtered through a 0.2µm PTFE disposable filter (Whatman). The filtrate (20µl) is injected into the HPLC system (HP1100, Agilent) via an autosampler maintained at 4°C. The analytical column (Prodigy Phenyl-3, 4.6 x 150mm, particle size 5µm, (Phenomenex) is held at 30°C. Detection is by diode array, monitoring at 262, 280, and 370 nm. Observed peaks are scanned from 210-550 nm to obtain spectra. Chemstation software (Rev. A.8.03) was used to control the system and collect and analyse data.

Absorbance spectra (corrected for baseline spectrum) and retention time of peaks are compared with those of commercially available flavonol standards to determine whether the plant tissue is active in flavonol biosynthesis.

It is preferred that a plant or the part thereof that is active in flavonol biosynthesis contains at least 10mg/kg fresh weight of flavonol, preferably at least 100mg/kg more preferred at least 1000mg/kg, most preferred from 1000 to 10000mg/kg.

A 'functional equivalent' nucleotide sequence is any sequence which encodes a protein which performs the same biological function.

According to another embodiment, a functionally equivalent nucleotide sequence shows at least 50% identity to the respective DNA sequence. More preferably a functionally equivalent DNA sequence shows at least 60%, more preferred at least 75%, even more preferred at least 80%, even more preferred at least 90%, most preferred 95-100% identity, to the respective DNA sequence (DNAStar MegAlign Software Version 4.05 and the Clustal algorithm set to default parameters)

According to a further preferred embodiment a functionally equivalent sequence shows not more than 5 base pairs difference to

the respective DNA sequence, more preferred less than 3, e.g. only 1 or 2 base pairs different.

According to another embodiment a functionally equivalent sequence is capable of hybridising under low stringent (2xSSC, 0.1%SDS at 25°C for 20min) conditions to the respective sequence, more preferably a functionally equivalent sequence is capable of hybridising under medium stringent conditions (1xSSC, 0.1%SDS, 25°C for 20min), further preferred a functionally equivalent sequence is capable of hybridising under high stringent conditions (0.1xSSC, 0.1%SDS, 25°C for 20min).

Preferably an equivalent DNA sequence is capable of transcription and subsequent translation to an amino acid sequence showing at least 50% identity to the amino acid sequence encoded by the respective DNA sequence. More preferred, the amino acid sequence translated from an equivalent DNA sequence has at least 60%, more preferred at least 75%, even more preferred at least 80%, even more preferred at least 90%, most preferred 95-100% identity to the amino acid sequence encoded by the respective DNA sequence (DNAStar MegAlign Software Version 4.05 and the Clustal algorithm set to default parameters.)

Brief description of the invention

It has now been found that novel plants which comprise significantly increased levels of daidzein and/or daidzein derivatives may be provided by a plant or part thereof which has both active anthocyanin and flavonol pathways and has been genetically modified to increase the enzyme activity of CHR and IFS therein.

It is a therefore a first object of the invention to provide a genetically modified plant or part thereof comprising daidzein and/or derivatives thereof, wherein said plant or part thereof is active in flavonol and anthocyanin biosynthesis and comprises one

or more nucleotide sequences encoding chalcone reductase and one or more nucleotide sequences encoding isoflavone synthase.

Particular advantage has been found to result from genetically modifying said plant to also increase the activity CHI, wherein production of daidzein in the tissues concerned showed a 90 fold increase over the modification to increase CHR and IFS activities alone. This additional benefit has been found to be dependent on the selection of a CHI isoform which is capable of catalysing the conversion of 4,2',4'-trihydroxychalcone to 7,4'-dihydroxyflavanone (liquiritigenin). Suitable CHI's are obtained from leguminous plants. At some 15 times (on a dry weight basis) the level of daidzein present in conventional soy this result represents a significant improvement to the art. This result is surprising and clearly demonstrates a synergy from a combined increase in these three enzyme activities where active anthocyanin and flavonol pathways are present in a plant.

A first embodiment of the invention therefore comprises a genetically modified plant or part thereof as described above, further comprising one or more nucleotide sequences encoding a chalcone isomerase capable of catalysing the conversion of 4,2',4'-trihydroxychalcone to 7,4'-dihydroxyflavanone.

A further embodiment of the invention comprises a genetically modified plant or part thereof as described above wherein said one or more nucleotides sequences comprise sequences according to sequence identification numbers 1 and 3, or functional equivalents thereof.

In the preferred instance where the synergistic advantage of an additional CHI increase is sought, the invention relates to an embodiment wherein said one or more nucleotides sequences comprises sequences according to sequence identification numbers 1, 3 and 5, or a functional equivalents thereof.

In a further embodiment the invention relates to a genetically modified plant or part thereof as described above wherein said plant or part thereof is selected from the group comprising, but not restricted to, tobacco, *Lactuca sp.*, broccoli, asparagus, red cabbage, potato, spinach, rhubarb, red onion, shallot, aubergine, radish, Swiss chard, purple basil, watermelon and berries.

Plants or parts thereof modified in accordance with the invention to increase their content of daidzein and/or daidzein derivatives are particularly advantageous for providing health benefits associated with increased dietary uptake of isoflavones. Plants or parts thereof may therefore be used either in their natural state or prepared as a extract to treat disease states or induce health benefits as a preventative agent or by counter acting the ageing process.

A second object of the invention therefore provides an extract of a plant as described above wherein said extract comprises daidzein or derivatives thereof.

A third object of the invention provides an extract as described above for use as a medicament. In a preferred embodiment and extract according to the invention may be used in the treatment and/or prevention of one or more conditions selected from the group comprising, osteoporosis; cancer; menopausal and post menopausal symptoms comprising hot flushes, anxiety, depression, mood swings, night sweats, headaches, unrinary incontinence; pre-menstrual syndromes comprising fluid retention, cylical mastalgia, dysmenorrhoea; heart disease atherosclerosis; hypertension; coronary artery spasm; high cholesterol; Alzheimer's disease; impaired cognitive function; inflammatory diseases comprising inflammatory bowel disease, ulcerative colitis, Crohn's disease; and rheumatoid arthritis.

Cosmetic benefits may also be gained from the use of an extract as described above in the treatment and/or prevention of one or more conditions selected from the group comprising sunlight induced

skin damage, skin wrinkling, loss of skin sensitivity, loss of skin firmness, acne, poor hair condition and baldness.

These medical and cosmetic benefits are also provided by the use of the genetically modified plants or parts thereof according to the invention. Uptake of the daidzein and/or its derivative may be via oral or topical applications.

For convenient dietary uptake of increased amounts of isoflavones a plant or part thereof according to the invention may suitably be incorporated into a food product or nutritional supplement. Hence, a further object of the invention provides for the use of a genetically modified plant or part thereof or of an extract as described in a food product or nutritional supplement.

It is to be noted that unlike conventional soy derived sources of dietary isoflavones, daidzein and the derivatives thereof as provided by plants according to the invention may be incorporated into food products without adversely affecting the flavour profile of such products. In this way an additional problem in the art is solved by the present invention.

A further object of the invention is therefore to provide a food product comprising a genetically modified plant or part thereof according to the description above. Preferably a food product according to the invention will be frozen to allow the content of daidzein and/or its derivatives to remain stable on storage.

In a most preferred embodiment a food product according to the invention is selected from the group comprising pre-packed mixed salads, soups, spreads, sauces, fruit/cereal bars and ice creams.

A nutritional supplement comprising an extract of a plant or part thereof as described above is also provided by the present invention.

It is a further object of the invention to provide a process for increasing the content of daidzein and/or derivatives thereof in a plant or part thereof, wherein said process comprises the steps;

(i) selecting a non-isoflavone producing plant wherein said plant or part thereof is active in anthocyanin and flavonol biosynthesis;
(ii) genetically modifying said plant to increase the activity of chalcone reductase and isoflavone synthase in said plant or part thereof.

In a first embodiment, the process of the invention further comprises genetically modifying said plant or part thereof to increase the activity of a chalcone isomerase wherein said chalcone isomerase is capable of catalysing the conversion of 4,2',4'-trihydroxychalcone to 7,4'-dihydroxyflavanone. In this way the process achieves the synergistic increase in the content of daidzein and/or its derivatives.

A further embodiment wherein the activity of chalcone reductase and isoflavone synthase are to be increased comprises a process as disclosed above wherein one or more nucleotide sequences according to sequence identification numbers 1 and 3, or functional equivalents thereof are stably integrated into the genome of said plant.

To achieve the desired increase in activity of chalcone isomerase a preferred embodiment comprises a process as disclosed above additionally comprising stably integrating into the genome of said plant one or more nucleotide sequence according to sequence identification number 5, or functional equivalents thereof.

In a most preferred embodiment the process according to the invention relates to a plant or part thereof selected from the group comprising, but not resticted to, tobacco, *Lactuca sp.*, broccoli, asparagus, red cabbage, potato, spinach, rhubarb, red

onion, shallot, aubergine, radish, Swiss chard, purple basil, watermelon and berries such as strawberries.

Detailed description of the invention

A sequence encoding a biosynthetic enzyme for increasing the tissue content of daidzein and/or daidzein derivatives may be a genomic or cDNA clone, or a sequence which in proper reading frame encodes an amino acid sequence which is functionally equivalent to the amino acid sequence of the biosynthetic gene encoded by the genomic or cDNA clone. A functional derivative can be characterised by an insertion, deletion or a substitution of one or more bases of the DNA sequence, prepared by known mutagenic techniques such as site-directed mutagenesis or derived from a different species.

For the performance of the present invention any nucleotide sequences encoding an enzyme with the biological function of a chalcone reductase, isoflavone synthase or chalcone isomerase may be used in the transformation of a suitably selected plant to increase these enzyme activities with said plant or part thereof.

Biological function of a nucleotide sequence encoding a chalcone reductase can be assessed by a standard assay (Welle et al., 1988 FEBS letter 236:221-225; Welle et al., 1991 Eur J Biochem 196:423-430; Welle and Schroder, 1992 Arch. Biochem. Biophys 293:377-381). To obtain protein, the nucleotide sequence is sub-cloned into a prokaryotic expression vector, such as pTZ19R (Pharmacia), and transformed into *Escherichia coli*. Selected *E.coli* clones harbouring the nucleotide sequences of interest are grown to a culture density of A_{600}=0.6-1 before inducing expression with 1mM isopropyl β-*D*-thiogalctopyranoside (IPTG) for 2.5 hours. Following induction, bacteria are harvested by centrifugation and resuspended in 0.1M potassium phosphate, 0.6mg/ml lysozyme and 1.2M EDTA and placed on ice for 45min to lyse. The lysate is centrifuged at 16000*g* for 20min and an aliquot of supernatant used in the chalcone reductase assay.

Chalcone reductase activity is assayed in a final volume of 120 µl, comprising 80µl chalcone reductase protein extract, 10µmol potassium phosphate pH 5.0, 0.12µmol NADPH, 1nmol 4-coumaroyl CoA, 1.5nmol [2-^{14}C] malonyl-CoA, 22.2 fkat pure soybean CHS (~3µg). Reactions are run for 60min at 30°C before the reaction products are extracted in 200µl ethyl acetate. The organic phase is separated by centrifugation, concentrated *in vacuo* and separated by thin layer chromatography using 15% acetic acid (presence of chalcone isomerase) or $CHCl_3$/acetic acid/water (10:9:1) (absence of chalcone isomerase). The identity of 6'-deoxychalcone is established by co-chromatography with a reference sample.

Suitable CHR encoding sequences already known in the art comprise; Alfalfa (Medicago sativa): accession numbers CHR1a - X82366, CHR1b - X82367, CHR2a - X82368, CHR7 - U13925, CHR12 - U13924; Chickpea (Cicer arietinum) accession number AB024989; Soybean (Glycine max) accession number X55730; Liquorice (Glycyrrhiza glabra) accession numbers CHRa - D86558, CHRb - D86559;

Alternatively suitable CHR encoding sequences may be isolated from other species. Sequence alignment of CHR's already known in the art, show two conserved regions Met-Pro-Val-Val-Gly-Met-Gly-Ser-Ala (Seq. ID No.7) and Ala-Ile-Ile-Glu-Ala-Ile-Lys-Gln (Seq. ID. No. 8) identified toward the 5' end of the coding sequence. Degenerate primers 327 and 328 (see figure 4) are designed to each of these coding sequences respectively. Sequences encoding CHR are isolated by polymerase chain reaction using primers 327 and 328 in conjunction with a dT_{17} primer and using a 3' cDNA library target. The resulting fragments were cloned into a pT7 vector and sequenced. Alignment of these sequences with those known in the art would allow provisional identification. To obtain full-length coding sequence, 5' and 3' sequence can be obtained using standard 5'RACE and 3'RACE procedures as disclosed in example 1 (1.3.3).

A nucleotide sequence encoding an enzyme with isoflavone synthase activity may also be determined by a standard assay, wherein yeast microsomes are prepared from control WHT1 and strains expressing a cytochrome P450 cDNA according to the methods of Pompon et al., (Methods Enzymol. 272, 51-64). The assay is carried out according to Jung et al., (Nature Biotech 2000, vol 18 February 200, p208-212). The protein content of each microsome preparation is assayed using the Bradford protein micro assay (Bio-Rad. Hecules. CA). From 30 to 150 µg of microsomal proteins are incubated at room temperature in 80 mM K_2HPO_4, 0.5 mM glutathione. 20% (wt/vol) sucrose, pH 8.0 with 100 µM naringenin or 100 µM liquiritigenen substrate and 40 nmol of NADPH added per each 100 µl of final reaction volume. Following incubation, reactions are extracted with ethyl acetate. Samples assayed on a Hewlett-Packard 1100 series HPLC system using either a LiChrospher RP-C18 column (5m 250 x 3 mm) or a Phenomenexz Luna C18 (2) column (3u; 150 x 4.6 mm).

On the first column samples in ethyl acetate of candidate cDNA assays are isocratically separated for 5 min employing 65% methanol as a mobile phase. For the second column samples are evaporated and resuspended in 80% methanol and then separated using a 10 min linear gradient from 20% methanol/80% 10 mM ammonium acetate, pH 8.3 to 100% methanol at a flow rate of 1 ml min^{-1} or using 65% methanol as mobile phase for isocratic elution. Genistein and daidzein are monitored by the absorbance of 260 nm. Using authentic naringinen, liquiritigen, genistein and daidzein (Indofine Chemical, Somerville NI) dissolved in ethanol as standards for calibration peak areas are converted to nanograms.

To confirm the identity of genistein and daidzein, samples are evaporated and resuspended in 25% acetonitrile in water and assayed on a Hewlett-Packard/Micromass LC/MS by running 25 µl on a Zorbax Eclipse XDB-C8 reverse-phase column (3 x 150 mm 3.5µm) isocratically with 25% solvent B (0.1% formic acid in acetonitrile) in solvent A (0.1% formic acid in water). Mass

spectrometry is done by electrospray scanning from 200 to 400 m/e, using -6 volt cone voltage. The diode array signals were monitored between 200 and 400 nm in both instruments.

Suitable IFS sequences already known in the art include Mung Bean accession number AF195807; Red Clover accession number AF195811; and Snow Pea accession number AF195812.

Alternatively suitable IFS cDNAs may be isolated from other species. Jung et al. (Nature Biotech 2000, vol 18 February 200, p208-212) describe how mung bean sprouts and snow pea sprouts were obtained from the grocery store. Seeds for alfalfa, red clover, white clover, hairy vetch and lentil can be obtained from Pinetree Garden Seeds (New Gloucester, ME) seeds for lupine cv. Russel Mix were obtained from Botanical Interests (Boulder, CO), and seeds for sugarbeet were obtained from a commercial source.

Seedlings were grown and RNA prepared using TRIzol Reagent (Gibco BRL) and first-strand cDNA was prepared as described above. OligodT was used as the reverse transcription primer in all cases except with white clover for which random hexamers were used as the reverse transcription primer: Polymerase chain reaction amplifaction was carried out using Advantage-GC cDNA polymerase mix (Clontech) using primer set one 5'ATGTTGCTGGAACTTGCACTT-3' (Seq ID. No. 9) and 5'TTAGAAAGGAGTTTAGATGCAACG-3' (Seq. ID. No. 10) or the nested primer set two: 5'TGTTTCTGCATTGCGTCCCAC-3' (Seq. ID. No. 11) and 5'-CCGATCCTTGCAAGTGGAACAC-3'(Seq. ID. No. 12)as follows: Mung bean and red clover PCR products amplified using primer set one were cloned directly into pCR2.1.

For white clover, lentil, hairy vetch, alfalfa, lupine, and beet a first PCR with primer set one was followed by a second primer set two, and the resulting fragments cloned. For snow pea, a first PCR with primer set one was followed by a second PCR with high annealing temperature (60°C) using primer set one. The expected size product was gel purified and used as a template in a third

PCR with the high annealing temperature and primer set one. The resulting product was cloned into pCR2.1. All PCR fragments in pCR2.1 were sequenced. All alignments were carried out using DNAStar MegAlign software version 4.05 and the Clustal algorithm set to default parameters.

The coding regions for accession numbers AF195807 (mung bean). AF195811 (red clover), and AF195812 (snow pea) were amplified and cloned into pRS315-gal using "gap repair" and microsomes were produced and assayed as described above.

A nucleotide sequence encoding an enzyme with chalcone isomerase activity capable of catalysing the conversion of 4,2',4'-trihydroxychalcone to 7,4'-dihydroxyflavanone may be determined by a standard assay (Dixon et al., 1982 Biochem. Biophys Acta 715: 25-33; Mol et al., 1985 Phytochemistry 24: 2267-2269, Terai et al., 1996 Protein Expression and Purification 8:183-190). To obtain protein, the nucleotide sequence is sub-cloned into a prokaryotic expression vector, such as pET vectors (Invitrogen), and transformed into *Escherichia coli*. Selected *E.coli* clones harbouring the nucleotide sequences of interest are grown to a culture density of A_{600}=0.6-1 before inducing expression with 1mM isopropyl β-*D*-thiogalctopyranoside (IPTG) for 2.5 hours.

Following induction, bacteria are harvested by centrifugation and resuspended in 0.1M potassium phosphate, 0.6mg/ml lysozyme and 1.2M EDTA and placed on ice for 45min to lyse. The lysate is centrifuged at 16000*g* for 20min and an aliquot of supernatant used in the chalcone isomerase assay.

Chalcone isomerase activity is assayed in a final volume of 1ml, comprising either 18.4µm tetrahydroxychalcone (naringenin chalcone) or 12.7µg trihydroxychalcone (isoliquiritigenin) substrate, chalcone isomerase protein extract, 5% (w/v) bovine serum albumin and 0.1M potassium phosphate buffer (pH5.8). Chalcone isomerase

activity against both tri- and tetra-hydroxychalcone substrates is detected by a decrease in absorption at 385nm.

Suitable CHI sequences already known in the art comprise those derived from; French bean (*Phaseolus vulgaris*) accession number X16470; Kudzu vine (*Pueraria montana var. lobata*): accession number D63577; Soybean (*Glycine max*): accession number AF276302; Alfalfa (*Medicago sativa*): accession number M910079; Garden Pea (Pisum sativum): accession number U03433.

Alternatively the well-established correlation between CHI function and structure enables suitable CHI sequences to be isolated from other sources. Numerous cloning strategies have been shown in the art to be effective at isolating CHI cDNAs and may be adopted by the person skilled in the art to identify alternative CHI encoding sequences.

Shirley, B.W., et al., (Plant Cell, Vol. 4, 333-347 1992,) describes a PCR based approach to obtaining CHI cDNA from Arabidopsis wherein the identification of consensus sequences for primer design as well as PCR reaction conditions are disclosed. Sparvoli, F. et al., (Plant Mol. Biol. 24: 743-755, 1994) describes the cloning of CHI from a cDNA library by using heterologous Antirrhinum CHI cDNA probes. Grotewold E. et al., (Mol. Gen. Genet. (1994) 242: 1-8,) describes the isolation and characterisation of a maize gene encoding CHI, the cloning strategy and suitable primers.

The literature outlined above clearly demonstrates that corresponding CHI sequences from other plants; alternative cloning strategies for other CHI genes; knowledge of consensus sequences for the generation of primers; appropriate PCR conditions are known in the art. The person skilled in the art is therefore able to identify and use alternative CHI sequences for the transformation according to the present invention.

Gene constructs according to the invention either comprise one or more nucleotide sequences encoding chalcone reductase and isoflavone synthase, or comprise one or more nucleotide sequences encoding chalcone reductase, isoflavone synthase and chalcone isomerase depending on the magnitude of increase sought.

The gene sequences of interest will be operably linked (that is, positioned to ensure the functioning of) to one or more suitable promoters which allow the DNA to be transcribed. Suitable promoters, which may be homologous or heterologous to the gene (that is, not naturally operably linked to a gene encoding an enzyme for flavonoid biosynthesis), useful for expression in plants are well known in art, as described, for example, in Weising *et al.*, (1988) Ann. Rev. Genetics 22:421-477. Promoters for use according to the invention may be inducible, constitutive, or tissue-specific or have various combinations of such characteristics.

Useful promoters include, but are not limited to constitutive promoters such as carnation etched ring virus (CERV) promoter, cauliflower mosaic virus (CaMV) 35S promoter, or more particularly the enhanced cauliflower mosaic virus promoter, comprising two CaMV 35S promoters in tandem (referred to as a "Double 35S" promoter). These would have the effect of increasing isoflavonoid levels throughout a plant.

Accordingly, the invention provides in a further aspect a gene construct in the form of an expression cassette comprising as operably linked components in the 5'-3' direction of transcription, one or more units each comprising a suitable promoter in a plant cell, a plurality of nucleotide sequences selected from the group comprising sequences encoding a CHR and IFS and a suitable transcriptional and translational termination regulatory region. More preferably said group comprises sequences encoding CHR, IFS and a CHI capable of catalysing the conversion of 4,2',4'-trihydroxychalcone to 7,4'-dihydroxyflavanone.

The promoter and termination regulatory regions will be functional in the host plant cell and may be heterologous or homologous to the plant cell and the gene. Suitable promoters which may be used are described above.

The termination regulatory region may be derived from the 3' region of the gene from which the promoter was obtained or from another gene. Suitable termination regions, which may be used, are well known in the art and include *Agrobacterium tumefaciens nopaline synthase* terminator *(Tnos)*, *Agrobacterium tumefaciens mannopine synthase* terminator *(Tmas)*, the rubisco small subunit terminator (*TrbcS*) and the *CaMV 35S* terminator *(T35S)*. Particularly preferred termination regions for use according to the invention include the *Tnos* and *TrbcS* termination regions.

Such gene constructs may suitably be screened for activity by transformation into a host plant via *Agrobacterium tumefaciens* co-transformation and screening for daidzein levels.

Conveniently, the expression cassette according to the invention may be prepared by cloning the individual promoter/gene/terminator units into a suitable cloning vector. Suitable cloning vectors are well known in the art, including such vectors as pUC (Norrander *et al.*, (1983) Gene 26:101-106), pEMBL (Dente *et al.*, (1983) Nucleic Acids Research 11:1645-1699), pBLUESCRIPT (available from Stratagene), pGEM (available from Promega) and pBR322 (Bolivar *et al.*, (1977) Gene 2:95-113). Particularly useful cloning vectors are those based on the pUC series. The cloning vector allows the DNA to be amplified or manipulated, for example by joining sequences. The cloning sites are preferably in the form of a polylinker, that is a sequence containing multiple adjacent restriction sites, to allow flexibility in cloning.

Preferably the DNA construct according to the invention is comprised within a vector, most suitably an expression vector

adapted for expression in an appropriate host (plant) cell. It will be appreciated that any vector which is capable of producing a plant comprising the introduced DNA sequence will be sufficient.

Suitable vectors are well known to those skilled in the art and are described in general technical references such as Pouwels *et al.*, Cloning Vectors. A laboratory manual, Elsevier, Amsterdam (1986). Particularly suitable vectors include the Ti plasmid vectors.

Transformation techniques for introducing the DNA constructs according to the invention into host cells are well known in the art and include such methods as micro-injection, using polyethylene glycol, electroporation, or high velocity ballistic penetration. A preferred method for use according to the present invention relies on *Agrobacterium tumefaciens* mediated co-transformation.

After transformation of the plant cells or plant, those plant cells or plants into which the desired DNA has been incorporated may be selected by such methods as antibiotic resistance, herbicide resistance, tolerance to amino-acid analogues or using phenotypic markers.

Various assays within the knowledge of the person skilled in the art may be used to determine whether the plant cell shows an increase in gene expression, for example, Northern blotting or quantitative reverse transcriptase PCR (RT-PCR). Whole transgenic plants may be regenerated from the transformed cell by conventional methods. Such transgenic plants having improved daidzein levels may be propagated and crossed to produce homozygous lines. Such plants produce seeds containing the genes for the introduced trait and can be grown to produce plants that will produce the selected phenotype.

Plants or parts thereof which have been modified in accordance with the present invention may be the used as a source of daidzein and/or one or more of its derivatives in the form of an enriched extract or a substantially pure form.

Food products which comprise the plants, plant parts or extracts thereof in accordance with the present invention enable the consumer to take full advantage of the health benefits associated with increased isoflavone uptake while at the same time avoiding the adverse flavour associated with soy derived isoflavones in the prior art.

Salad leaves are particularly suited to genetic transformation by the process of the invention and therefore species of lettuce (*Lactuca sp.*) such as *Lactuca sativa* e.g. `Red Oak Leaf', `Red Leprechaun'; *Lactuca sativa* group Butterhead lettuce e.g. Mira, Redcross; *Lactuca sativa* group Cos lettuce e.g. `Romaine Red Cos', Four Seasons Red', Seville; *Lactuca sativa* group Crisp lettuce e.g. `Red Salad Bowl', Red Grenoble'; *Lactuca sativa* group Cutting lettuce e.g. `Lollo Rosso', Revolution transformed in accordance with the present invention provide a ideal means of supplementing dietary needs and may be provided washed and pre-packed to the consumer.

Fruit containing snack bars or breakfast cereals provide a convenient means of supplementing the human diet with isoflavones. Fruit pieces derived from a plant according to the invention are suitably dried to from 10 to 90%, preferably 20 to 60%, most preferably about 40% of their fresh weight to give shelf stability and incorporated into a bar or cereal product.

Fruits with high levels of daidzein and/or daidzein derivatives in accordance with the invention are also be ideally incorporated into yoghurts and ice creams or to flavour fruit drinks.

Suitable fruits for these food products would include raspberries, strawberries, blackcurrants, red currants, blueberries and blackberries.

Plants or parts thereof which have been genetically modified in accordance with the present invention may also provide a source of an extract rich in daidzein and/or its derivatives or a purified form thereof for inclusion in products such as nutritional supplements, calorie controlled drinks and low fat spreads.

A large body of evidence supports the cosmetic and medical health benefits that can be attributed to human dietary consumption of isoflavones and in particular daidzein. These include: activity as both estrogenic and anti-estrogenic agents (Coward et al., 1993; Martin, et al., 1996); anticancer effects associated with phytoestrogenic activity (Lee et al., 1991; Adlercreutz et al., 1991); anticancer effects associated with inhibition of several enzymes including DNA topoisomerase and tyrosine protein kinase (Akiyama, et al., 1987; Uckun, et al., 1995); suppression of alcohol consumption (Keung and Vallee, 1993; Keung et al., 1995); antioxidant activity (Arora et al., 1998; Tikkanen et al., 1998); increasing bone remineralisation (Tomonaga et al., 1992; Draper et al., 1997); and beneficial cardiovascular effects (Wagner et al., 1997).

The present invention may be more fully understood by reference to the accompanying figures in which:

Figure 1: shows the pea chalcone reductase DNA sequence (SEQ ID No. 1) and its corresponding protein sequence (SEQ ID No. 2).

Figure 2: shows the soy isoflavone synthase DNA sequence (SEQ ID No.3) and its corresponding protein sequence (SEQ ID No. 4)

Figure 3: *Lotus corniculatus* chalcone isomerase DNA sequence (SEQ ID No. 5) and its corresponding protein sequence (SEQ ID No. 6)

Figure 4: provides primer sequences used in accordance with the invention.

Figure 5: illustrates Plasmid maps of pPV5LN, pPE2, pPE5, pPE9, pPE11, pPE15, pPE51, pPE120 and pPE125.

Figure 6: illustrates GC-MS analysis of tobacco petal extracts from representative tobacco transfomants, pPE120/24, pPE120/26 and pPE51 spiked with an authentic daidzein standard. A. Peak with retention time corresponding with authentic daidzein (RT= 19.60) is present I pPE120/24 and pPE120/26 transformants. B. Selected ion monitoring of pPE120/24 and pPE51/9 spiked with an authenitc daidzein standard shows characteristic peaks in pPE120/24.

Figure 7: illustrates accumulation of daidzein in petal tissue from tobacco transformants harbouring constructs encoding chalcone reductase and isoflavone synthase (pPE120) activitites with controls (pPE51). Ethanol extracts from petals were hydrolysed and analysed by HPLC.

Figure 8: illustrates accumulation of daidzein in petal tissue from tobacco transformants harbouring constructs encoding chalcone reductase, chalcone isomerase and isoflavone synthase (pPE125) activitites with controls (pPE51). Ethanol extracts from petals were hydrolysed and analysed by HPLC.

Example 1: cDNA cloning of chalcone reductase, chalcone isomerase and isflavone synthase and the generation and analysis of transgenic N. tabacum.

1.1 Plant material

All experiments can be performed using normally available tobacco (*Nicotiana tabacum*) genotypes as the starting material. *N.tabacum* cultivar SR1 is such a genotype. Plants of *N.tabacum* cultivar SR1 were grown in controlled temperature growth rooms with a 16-hour photoperiod at a temperature of 25°C.

1.2 Bacterial strains

Escherichia coli strain XL1-Blue: *rec*A1, *end*A1, *gyr*A96, *thi*-1, *hsd*R17, *sup*E44, *rel*A1 *lac* [F'*pro*AB lacl^{q}ZΔM15 Tn*10* (Tet^r)](Stratagene Europe, The Netherlands). Transformation of E. coli XL1-Blue was performed using the method of Hanahan (1983). *Agrobacterium tumefaciens* LBA4404 (Hoekema, 1985). Transformation of *Agrobacterium tumefaciens* LBA4404 was performed according to Shen and Forde (1989).

1.3 Gene cloning

1.3.1 Total RNA isolation

RNA was isolated from *Lotus corniculatus* (Lotus), *Glycine max* (soybean), *Pisum sativum* (pea) and *Medicago sativa* (alfalfa) leaf tissue using a Purescript RNA isolation kit (Pharmacia) according to manufacturer's instructions.

1.3.2 cDNA synthesis

5' cDNA library construction: 2µg of RNA isolated from either Lotus, soybean, pea or alfalfa tissue was heated to 65°C for 10 minutes, then snap cooled on ice. The RNA was reverse transcribed

in a 20µl reaction for 90 minutes at 42°C using 10 units of stratascript (Gibco-BRL) in 1 x rt buffer (Gibco BRL), 30mM dNTPs (dATP, dCTP, dTTP, dGTP) (Pharmacia), 0.1M DTT, 1U/µl RNasin (Roche) and 50 pmoles random hexamers. The random primed cDNA was then purified using a Gibco-BRL pcr purification kit (according to manufacturer's instructions). The purified cDNA was then poly A tailed in 50µl of 1x tailing buffer (Roche), 1mM dATP (Roche), 1 unit terminal transferase (Roche) at 37°C for 5 minutes then denatured at 80°C for 15 minutes.

3′ cDNA library construction: 2µg of RNA isolated from either Lotus, soybean, pea or alfalfa tissue was heated to 65°C for 10 minutes, then snap cooled on ice. The RNA was reverse transcribed in a 20µl reaction for 90 minutes at 42°C using 10 units of stratascript in 1 x RT buffer 30mM dNTPs, 0.1M DTT, 1U/µl Rnasin and 5 pmoles oligo dt_{17}.

1.3.3 PCR amplification

Library PCR amplification: 50ng of 3′ cDNA was PCR amplified in 50µl of 1x PCR buffer (Roche), 20mM dNTPs 25 pmoles 5′ primer, 25 pmoles 3′ primer, 2.5 units *Taq* DNA polymerase (Roche), 0.25 units *pfu* turbo DNA polymerase (Stratagene). Cycling conditions were; 30 cycles of 94°C for 30 seconds, 55°C for 30 seconds, 72°C for 2 minutes, using a Perkin Elmer PCR machine. The initial denaturing step (94°C) was extended to 2 minutes.

Vector PCR amplification: 1ng of a vector was PCR amplified in 50µl of 1x PCR buffer (Stratagene), 20mM dNTPs 25 pmoles 5′ primer, 25 pmoles 3′ primer, 5 units *pfu* turbo DNA polymerase. Cycling conditions were; 30 cycles of 94°C for 30 seconds, 55°C for 30 seconds, 72°C for 2 minutes. The initial denaturing step (94°C) was extended to 2 minutes.

5' Rapid Amplification of cDNA Ends (5'race): 50ng of 5' cDNA was complemented in 50µl of 1x PCR buffer, 20mM dNTPs, 5 pmoles oligo dt_{17}, 1.25 units *Taq* DNA polymerase, 0.125 units *pfu* turbo DNA polymerase. Conditions were 94°C for 2 minutes, 42°C for 2 minutes, 72°C 45 minutes. The cDNA was amplified by adding the following; 25 pmoles 5' R_O primer, 25 pmoles primer R_O, 1.25 units *Taq* DNA polymerase, 0.125 units *pfu* turbo DNA polymerase to the reverse transcription reaction. Cycling conditions were; 30 cycles of 94°C for 30 seconds, 55°C for 30 seconds, 72°C 2 minutes. The initial denaturing step was extended to 2 minutes. 1µl of this PCR reaction was re-amplified in 50µl of 1x PCR buffer, 20mM dNTPs, 25 pmoles 5'R_O primer, 25 pmoles R_O primer, 2.5 units *Taq* DNA polymerase, 0.25 units *pfu* turbo DNA polymerase. Cycling conditions were; 30 cycles of 94°C for 30 seconds, 55°C for 30 seconds, 72°C 30 seconds. The initial denaturing step was extended to 2 minutes.

3' Rapid Amplification of cDNA Ends (3'race): 50ng of 3' cDNA was amplified in 50µl of 1x PCR buffer, 20mM dNTPs, 25 pmoles 5'R_O primer, 25 pmoles primer R_O, 2.5 units *Taq* DNA polymerase, 0.25 units *pfu* turbo DNA polymerase. Cycling conditions were; 30 cycles of 94°C for 30 seconds, 55°C for 30 seconds, 72°C 2 minutes. The initial denaturing step was extended to 2 minutes. 1µl of this PCR reaction was re-amplified with 5'R_I primer and R_I as before with cycling conditions: 30 cycles of 94°C for 30 seconds, 55°C for 30 seconds, 72°C I minute. The initial denaturing step was extended to 2 minutes.

1.3.4 Isolation and cloning of amplified products

Fragments generated by PCR were analysed on an ETBr-1.2% agarose TBE (45mM Tris-borate, 1mM EDTA) gel. DNA fragments were isolated from the gel using a Gibco-BRL gel extraction kit according to manufacturer's instructions and cloned into a pT7 TA cloning vector (Novagen).

1.3.5 Digesting DNA with restriction enzymes

PCR amplifications or 2µg of plasmid DNA were digested with 10 units of each appropriate restriction enzyme (Roche) in the recommended buffer at 37°C for 2 hours. Digests were separated on EtBr-1.2% agarose TBE gel: The desired fragments were excised and purified using the Gibco-BRL gel extraction kit according to manufacturer's instructions.

1.3.6 Construction of synthetic linkers

1µg of sense and anti-sense oligonucleotides were annealed together by heating to 94°C for 5 minutes in 1 x ligation buffer and then cooled to room temperature over a period of 30 minutes.

1.3.7 De-phosphorylation of DNA fragments

Vector DNA fragments were incubated in 50µl of 1x sip (Roche) buffer with 0.5 units shrimp intestinal phosphorylase (Roche) at 37°C for 15 minutes and then denatured at 80°C for 5 minutes.

1.3.8 Sub-cloning into vectors

DNA fragments of interest were ligated into appropriate vectors in a ratio of 5:1 in a final volume of 20µl containing 1x ligation buffer (Roche), 2 units of T4 DNA ligase (Roche) at 4-8°C for 16 hours.

1.3.9 Preparation and transformation of competent *E. coli*

To prepare competent cells a culture of XL1-Blue (from a single colony) was grown up overnight at 37°C, 225 r.p.m. in 10ml Lennox broth containing 12.5µg/ml tetracycline. 1ml from this overnight culture was transferred into 100mls of fresh, pre-warmed, Lennox broth and cultured for a further 2 hours until the OD_{600} was in the range 0.3 to 0.6. The cells were then recovered by centrifugation

at 4500g for 10 minutes at 4°C. The cells were washed in 50ml 100mM $CaCl_2$, before resuspending in a final volume of 5ml 100mM $CaCl_2$. The cells were then placed on ice for 1 hour.

Transformations were performed as follows: One-fifth (4µl) of the ligation reaction was added to 200µl competent cells. The mixture was incubated on ice for 30 minutes then heat shocked at 42°C for 40 seconds. 300µl of 2YT was then added to the mixture before incubating at 37°C, 225 r.p.m. for 30 minutes. The transformations were then plated out on Lennox agar containing 100µg/ml carbenicillin or 50µg/ml kanamyicin and incubated at 37°C overnight.

1.3.10 Identification and screening of E. *coli* recombinants

Positive transformants were identified by amplifying DNA from a single colony in a 50µl reaction containing the following mixture, 1 x pcr buffer, 0.2mM dNTPs, 25 pmoles 5′ primer, 25 pmoles 3′ primer, 1.25 units *Taq* DNA polymerase. Cycling conditions were 94°C for 30 seconds, 55°C for 30 seconds, 72°C 1 minute, for 30 cycles. The initial denaturing step was extended to 2 minutes. The pcr amplifications were then analysed on EtBr-1.2% agarose TBE gels.

1.3.11 Extraction and purification of plasmid DNA

Selected colonies were grown up overnight in 50mls of 2TY broth containing either 100µg/ml carbenicillin or 50µg/ml kanamycin (as appropriate) at 225 r.p.m. at 37°C. The cells were recovered by centrifugation at 4500g for 10 minutes at 4°C. The bacterial pellet was resuspended in 4ml of solution 1 (25mM Tris.Cl pH8.0, 10mM EDTA), then 8ml of solution 2 (0.2N NaOH, 1% SDS) was added, and left at room temperature for 5 minutes to lyse the cells. 6ml of ice-cold solution 3 (3M potassium, 5M acetate) was added and the mixture incubated on ice for 15 minutes. The bacterial lysate was then centrifuged at 15000g, 4°C for 20 minutes, and the

supernatant was filtered through 4 layers of miracloth (CalBiochem). 10mls of Isopropanol was added to precipitate the DNA and the precipitate spun for 15 minutes at 15000g, at room temperature. The pellet was resuspended in 1ml of TE (10mM Tris.Cl pH7.6, 1mM EDTA) with 10µg/ml RNase A and incubated at 50°C for 30 minutes to remove contaminating RNA. The solution was extracted twice with an equal volume of phenol/chloroform (1:1) then once with an equal volume of chloroform. The DNA was re-precipitated with 0.1 volume 3M NaAc pH5.2 and 0.7 volume isopropanol, then spun for 5 minutes at 10000g, at room temperature. The pellet was washed in 70% ETOH then air dried and resuspended in TE at a final concentration of 1µg/µl.

1.4 Vector construction

Expression vectors were generated containing chalcone reductase (CHR), chalcone isomerase (CHI) and isoflavone synthase (IFS) cDNA's from *Pisum sativum, Lotus corniculatus* and *Glycine max* respectively. CHR, CHI and IFS transgenes were placed under the control of the double 35s promoter to give high levels of expression in tobacco tissues.

1.4.1 Construction of plasmid pPV5LN

To construct pPV5LN, pUC19 was modified as follows. Firstly, plasmid pPV3 was constructed by removing the HindIII/EcoRI multiple cloning site from pUC19 and replacing it with a synthetic DNA fragment, destroying the original EcoRI and HindIII sites and introducing SgfI, HindIII, KpnI, EcoRI and XbaI restriction sites. This synthetic fragment was constructed by annealing the oligonucleotides 624 and 625 (Figure 4). This resulted in plasmid pPV3.

The KpnI/EcoRI insert from pSJ30 containing the 2x35S-promoter sequence upstream of an ~1.9kb coding sequence, followed by the

Nos terminator sequence was ligated with pPV3 restricted with KpnI/EcoRI. This resulted in plasmid pPV5.

The ~1.9kb coding sequence was then removed from pPV5 as a SalI/SacI fragment and replaced by a synthetic DNA fragment introducing NcoI, NheI and MunI restriction sites, while leaving the original SalI/SacI sites intact. This synthetic fragment was constructed by annealing the oligonucleotides 626 and 627 (Figure 4). This resulted in plasmid pPV5L.

The sequence immediately 5' of the start codon ATG in pPV5L (CCACC) was replaced by the plant Kozak sequence TAAACC using PCR. Oligonucleotides 640 and 641 (Figure 4) were used to amplify the 189 bp 3' fragment of the 2x35S promoter from vector pCP031 (van Engelen et al., 1994), modifying the Kozak sequence via oligonucleotide 641. pCP031 and the amplified fragment were then restricted with HindIII/EcoRV and EcoRV/NcoI respectively before ligation with pPV5L restricted with HindIII-NcoI to replace the promoter. This resulted in plasmid pPV5LN (Figure 5).

1.4.2 Construction of plasmid pPE-2

To construct the plasmid pPE-2, the multiple cloning site of pPV5LN was modified by the insertion of three oligonucleotide adapters. First, oligonucleotides 331 and 332 (Figure 4) were annealed together and ligated with plasmid pPV5LN restricted with EcoRI-XbaI. This resulted in plasmid p5LNa. Next, the multiple cloning site from pPV5LN was amplified using oligonucleotides 248 and 191 (Figure 4) and the amplification product restricted with XbaI and EcoRI. This product was then ligated, in conjunction with the annealed product of oligonucleotides 333 and 334 (Figure 4) with p5LNa restricted with NcoI-EcoRI. This resulted in plasmid p5LNb. To construct plasmid pPE-2, oligonucleotides 329 and 330 were annealed together and ligated with plasmid p5LNb restricted with SfiI-HindIII. This resulted in plasmid pPE-2 (Figure 5).

1.4.3 Construction of plasmid pPE-5

To construct the plasmid pPE-5, the multiple cloning site of pSJ34 was modified by the insertion of an oligonucleotide adapter. First, oligonucleotides 337 and 338 (Figure 4) were annealed together and ligated with plasmid pSJ34 restricted with HindIII-EcoRI. This resulted in plasmid pPE-5 (Figure 5).

pSJ34 is a derivative of the binary vector pGPTV-Kan (Becker et al., 1992 Plant Mol. Biol. 20: 1195-1197) in which the BamHI site between the nptII selectable marker and the gene7 poly (A) signal was destroyed by 'filling-in' with klenow polymerase.

1.4.4 Construction of plasmid pPE-9 (2x35S+kozak-Lotus CHI-Tnos)

Lotus CHI cDNA was amplified from the lotus 3' and the 5' cDNA library using primers 160/323 and 160/321 respectively (Figure 4); the amplification products were then re-amplified using primers 198/324 and 198/322 respectively (Figure 4). The amplified fragments were separated by electrophoresis and products 5a.3.19 and 2.11 respectively were cloned into the vector pT7 and sequenced with primers 152 and 191 (Figure 4).

To verify the DNA sequence of the amplified fragments primers 386 and 387 were used to amplify the complete coding region of the CHI gene (in triplicate) from a lotus 3' cDNA library. The resultant fragments LCHI-A, LCHI-B and LCHI-C were cloned into vector pT7 and sequenced with primers 152 and 191. Clone LCHI-A was re-amplified with primers 386/403 and 402/387, the resultant fragments were digested with *NcoI-PstI* and *PstI-NheI* respectively and ligated into *NcoI-XbaI* opened PE-2 to create the vector PE-9 (2x35S+Kozak-Lotus CHI-tNOS in PE-2) (Figure 5).

1.4.5 Construction of plasmid pPE-11 (2x35S+kozak-Pea CHR-Tnos)

The chalcone reductase cDNA was amplified from a *Pisum sativum* leaf tissue 3' cDNA library using primers 384 and 385. The resulting 0.98kb product was ligated with the PCR cloning vector pT7Blue [Novagen] and the sequence verified before further sub-cloning.

The chalcone reductase sequence was then amplified from the pT7Blue vector using oligonucleotides 384/362, 363/398, 399/400 and 401/385 (Table 1). The resulting amplification products were restricted with NcoI-NarI, NarI-BamHI, BamHI-MunI, and MunI-NheI respectively before ligation with pPE-2 restricted with NcoI-XbaI. This resulted in plasmid pPE-11 (Figure 5).

1.4.6 Construction of plasmid pPE-15 (2x35S+kozak-Soy IFS-Tnos)

The isoflavone synthase cDNA was amplified from a *Glycine max* leaf tissue cDNA library using primers 339/340, 341/342, and 343/344 (Table 1). The resulting amplification products were restricted with NcoI-ApaI, ApaI-SalI, and SalI-NheI respectively before ligation with pPE-2 restricted with NcoI-XbaI. This resulted in plasmid pPE-15 (Figure 5).

1.4.7 Construction of plant transformation vector pPE-120 (CHR-IFS)

The single gene constructs described above were used to construct the plasmid pPE120 as follows. Plasmids pPE-11 and pPE-15 were restricted with SalI-EcoRI and HindIII-SalI respectively. The 2x35S+kozak-Pea CHR-Tnos and 2x35S+kozak-Soy IFS-Tnos fragments were then ligated with pPE-5 restricted with HindIII-EcoRI. This resulted in plasmid pPE120 (Figure 5).

1.4.8 Construction of plant transformation vector pPE-125 (CHR-CHI-IFS)

To construct the plant transformation vector pPE125, plasmids pPE-9 and pPE-120 were restricted with SalI. The resulting 2x35S+kozak-Lotus CHI-Tnos fragment (from pPE-9) was then ligated with SalI linearised pPE-120. This resulted in plasmid pPE-125 (Figure 5).

1.4.10 GPTV control plasmid

A GPTV-based binary plasmid, pPE51 (Figure 5), containing the double CaMV *35s* promoter and the *nos* poly(A) signal (*Pd35s- Tnos*) was used as control plasmid. This allows direct comparison between transformed control plants and plants containing the CHR, CHI and IFS constructs generated via a tissue culture procedure.
pPE51 was constructed by restricting pPE2 with EcoRI-HindIII. The 2x35S+kozak-Tnos fragment was then ligated with pPE5 restricted with HindIII-EcoRI. This resulted in plasmid pPE51 (Figure 5).

1.5 *A.tumefaciens* transformation

Binary plasmids of pPE120, pPE125, pPE130 and pPE51 were introduced into *Agrobacterium tumefaciens* strain LBA4404 by high voltage electroporation as described by Shen and Forde (1989). Briefly, electrocompetent cells of *A.tumefaciens* were prepared by inoculation of 50ml of 2xYT medium (Sambrook *et al.*, 1989) and culturing with shaking at 100rpm at 28°C until the culture reached an OD_{600} of 0.5-0.7. The cells were cooled on ice, harvested by centrifugation and the supernatant discarded. The cells were then washed successively in 50, 25, 1 and 1 ml of cold 10% (v/v) glycerol before re-suspension in 0.5ml 10% glycerol.

For transformation, 40µl of cells were transferred to a pre-cooled 0.2cm electroporation cuvette (Bio-Rad Laboratories). One µl of either pPE120 or pCJ102 plasmid DNA was mixed with the cell suspension on ice and an electric pulse applied immediately using a Gene Pulser with Pulse controller unit (Bio-Rad). For transformation, the field strength was 12.5 kV/cm, a capacitance

of 25μF and resistors of 400-600 ohms in parallel, giving time constants of 8-12msec. The cells were immediately transferred to 1ml 2xTY and shaken at 29°C for 2hours. Aliquots were then plated onto LB agar supplemented with kanamycin and incubated for 2-3 days at 29°C.

The presence and integrity of the plasmids in kanamycin resistant clones was established by PCR analysis using pPE120 (GPTV2 and 30035s; 340-GPTV1), pPE51 (30035S and GPTV2), pPE125 (GPTV2 and 30035s; 340-GPTV1; 248-403; 402-398), pPE130 (GPTV2-30035S) specific primers respectively (Figure 4).

1.6 Stable transformation of *Nicotiana tabacum* cv SR1

A.tumefaciens cells from PCR positive colonies were used to inoculate a 10 ml Lennox media broth containing kanamycin 50 :g/ml and rifampicin 50 :g/ml and incubated overnight with shaking (120rpm) at 29°C. The overnight culture was centrifuged at 3000*g* and the cell pellet resuspended in an equal volume of MS media (3% sucrose). Leaf segments were cut from young *Nicotiana tabacum L. cv. SR1* leaves from plants grown in tissue culture. The leaf segments were placed directly into the *A.tumefaciens* suspension and co-incubated for 10 minutes.

The leaf segments were then transferred, axial surface down, to feeder plates (10 per plate) and placed at 22°C for 2 days in low light. The leaf segments were then transferred, axial surface up, to tobacco shooting media supplemented with hormones, cefotaxime 500 :g/ml and kanamycin 50 :g/ml and placed in a growth room at 24°C with a 16hr photoperiod. After three weeks, callusing segments were transferred to fresh tobacco shooting media in vitro-vent [Melford Laboratories Ltd.] tissue culture vessels. Shoots were then excised from callused leaf segments and placed on tobacco shooting media without hormones containing cefotaxime 500 :g/ml and kanamycin 50 :g/ml.

Genomic DNA was isolated from shoots that had rooted and transgenic plants harbouring the constructs were selected following specific amplification of the CHR,CHI, IFS transgenes respectively.

Transgene positive plants were then potted up into a 50% perlite 50% compost mixture and placed in a propagator in a growthroom at 25°C with a 16h photoperiod (3000 lux). After 1 week the plants were removed from the propagator and subsequently potted up into 5-inch pots.

Petal tissue was harvested from each independent transformant and stored for subsequent analysis. When flowering had finished, each plant was cut-back and allowed to re-grow to form new flowers, from which seeds were harvested for propagation and analysis.

1.7 Extraction of flavonoids and isoflavonoids from tobacco tissues

Flavonoids and isoflavonoids were determined as their glycosides or as aglycones by preparing non-hydrolysed and hydrolysed extracts, respectively.

For extraction, tobacco petal tissues were harvested from fully open, mature flowers. To ensure representative analyses, all of the flowers (> 10 per plant) were harvested at a similar developmental stage from each pPE120, pPE125 and corresponding pPE51 (control) plants. The flower was fractionated to remove stamen, carpel and corolla tube tissue and the remaining petal tissue was then flash frozen in liquid nitrogen before being stored at -80°C. The petal tissues (>10) from each plant were then ground to a fine powder to ensure a homogeneous mix. An aliquot from this mixture was then extracted for 30min at room temperature (~22°C) in 80% (v/v) ethanol at 100mg/700µl. Following extraction, the cell debris was removed by filtration

through a 0.45µm Millex-HV filter unit (Millipore Corp, USA). The filtrate was stored at -20°C prior to HPLC analyses.

For hydrolysed extracts, 40µl of 12M HCl was added to 360 µl from each petal extract, before incubating at 90°C for 40 min.

Daidzin/genistin standards were hydrolysed under the same conditions as the petal extracts providing a control for the hydrolysis process.

1.8 Flavonoid and isoflavonoid analyses

1.8.1 HPLC conditions for flavonoid and isoflavonoid analysis

After hydrolysis, an aliquot from each extract was filtered through a 0.2µm PTFE disposable filter (Whatman). The filtrate (20µl) from was injected into the HPLC system (HP1100, Agilent) via an autosampler maintained at 4°C. The analytical column (Prodigy Phenyl-3, 4.6 x 150mm, particle size 5µm, (Phenomenex) was held at 30°C. Detection was by diode array, monitoring at 262, 280, and 370 nm. Observed peaks were scanned from 210-550 nm to obtain spectra. Chemstation software (Rev. A.8.03) was used to control the system and collect and analyse data.

Separation of flavonoid and isoflavonoid components within the extracts was performed using a gradient of acetonitrile in 1% acetic acid, at a flow rate of 0.8ml/min. The gradient of acetonitrile was: 15-37% linear in 22min, then 37-80% in 2min, before a hold at 80% for 2min. Then the acetonitrile was reduced from 80-15% in 2min and held at 15% for 2min prior to next injection.

Absorbance spectra (corrected for baseline spectrum) and retention time of peaks were compared with those of commercially available flavonoid and isoflavonoid standards.

Calibration curves for quercetin, kaempferol, genistein, daidzein, isoliquiritigenin and liquiritigenin were established to permit quantitation in the hydrolysed tobacco extracts. Levels were calculated on a fresh weight (µg/g F.wt.) basis. With the HPLC system and software used, detection limits in tobacco extracts was about 0.1µg/ml, corresponding with ~1.5µg/g fresh weight. Variation between replicate injections was less than 5%.

1.8.2 GC-MS conditions for flavonoid and isoflavonoid analysis

After hydrolysis, 5ml of 10 % Na_2SO_4 was added to an aliquot from each tissue extract before extraction with 2ml ethyl acetate. The sample was then centrifuged at 1600*g* for 1 min. The ethyl acetate layer was decanted to a fresh tube and evaporated to dryness under N_2 (<45°C).

Samples were dissolved in 30 µl pyridine and derivatised by heating with 20µl bis-trifluoroacetamide (BSTFA) at 45°C for 15 min., 1µl of sample was injected onto a CP-Sil 8 CB/MS (25m x 0.25mm x 0.25µm film) GC capillary column (Chrompack) through a splitless injector port at 280°C (Hewlett Packard 5890 gas chromatograph). The oven temperature was set at a linear temperature gradient from 100-320°C at 10°C/min with a helium gas flow rate of 1ml/min. The mass spectrum was monitored using a Hewlett Packard 5972A quadruple mass-selective detector set at 300°C (EI) and mass ranges of 175,184,383,398 Daltons for daidzein (selective ion mode); 228,399,371,486 Daltons for genistein (selective ion mode); (219, 307, 371, 457 and 472 daltons for isoliquiritigenin (selective ion mode) and 151, 179, 192, 235, 385, and 400 daltons for liquiritigenin (selective ion mode). In addition, mass ranges of 170-400 Daltons for daidzein, 130-480 Daltons for isoliquiritigenin, 130-410 Daltons for liquiritigenin and 180-490 for genistein were selected for full scan mode.

1.9 Accumulation of daidzein in transgenic N. tabacum ectopically expressing chalcone reductase and isoflavone synthase:

To determine whether ectopic expression of both chalcone reductase and isoflavone synthase in the non-leguminous plant *N.tabacum* was able to redirect flavonoid synthesis toward daidzein and/or genistein synthesis, the flavonoid and isoflavonoid profile of petal tissues was determined. This analysis was performed by HPLC using hydrolysed extracts of petal tissue from nineteen pPE120 and six pPE51 transformants.

In the HPLC analysis comparison between hydrolysed petal extracts from flowers of *N.tabacum* transformed with either pPE120 or pPE51 indicated that in several of the pPE120 transformants a small peak with the same retention time as the daidzein standard was detected. By contrast, this HPLC peak was not present in control (pPE51) transformants. To confirm our preliminary identification, this peak was collected from the HPLC and analysed using GC-MS assay. In addition, fractions with the corresponding retention time were collected from a typical pPE51 transformant and from a daidzein standard as controls.

GC-MS analysis showed that the retention time and the relative abundance of the measured ions (175, 184, 383, and 398 [M^+]) from the pPE120 fraction were similar to those from the authentic daidzein standard (Figure 6). Furthermore, the fraction from pPE51 showed no GC peak with a similar retention time or with a similar relative abundance of the measured ions confirming the absence of daidzein in the control transformants (Figure 6).

Quantitation, based on comparison with authentic standards showed that levels of daidzein accumulation in pPE120 petal tissues reached up to ~2.75µg/gFwt (Figure 7).

1.10 Daidzein accumulation in transgenic *N.tabacum* expressing chalcone reductase, isoflavone synthase and chalcone isomerase.

To determine whether concomitant expression of chalcone reductase and isoflavone synthase in conjunction with a legume chalcone isomerase in the non-leguminous plant *N.tabacum* was able to enhance the level of daidzein accumulation, the flavonoid and isoflavonoid profile of petal tissues was determined. This analysis was performed by HPLC using hydrolysed extracts of petal tissue from twelve pPE125 and six pPE51 transformants.

In the HPLC assay comparison between hydrolysed petal extracts from flowers of *N.tabacum* transformed with either pPE125 or pPE51 indicated that for several of the pPE125 transformants a peak with the same retention time as the daidzein standard was detected. By contrast, this peak was not present in control (pPE51) transformants. To confirm our preliminary identification, the peak corresponding to daidzein was collected from the HPLC and analysed using GC-MS assay. In addition, fractions with the corresponding retention time were collected from a typical pPE51 transformant and from a daidzein standard as controls.

GC-MS analysis showed that the retention time and the relative abundance of the measured ions (175, 184, 383, and 398 [M^+]) from the pPE125 fraction were similar to those from the authentic daidzein standard. Furthermore, the fraction from pPE51 showed no peak with a similar retention time or with a similar relative abundance of the measured ions confirming the absence of daidzein. Quantitation, based on comparison with authentic standards showed that levels of daidzein accumulation in pPE125 petal tissues reached up to 246.7µg/gFwt (~4934µg/gDwt) (Figure 8).

Example 2: Transformation of lettuce

Stable transformation of *Lactuca Sativa L.* cv Lollo Rossa, Bijou, Muscara & Revolution

A.tumefaciens cells from PCR positive colonies were used to inoculate a 10ml Lennox media broth containing kanamycin 50µg/ml and rifampicin 50 µg/ml and incubated overnight with shaking (120rpm) at 29°C. The overnight culture was centrifuged at 3000*g* and the cell pellet resuspended in an equal volume of UM media and a 1:10 (v/v) dilution used for transformation.

Cotyledons were cut from 7-day old *Lactuca Sativa L.* seedlings grown in tissue culture. The abaxial surface of the cotyledons was scored with a scalpel-blade before placing directly into the *A.tumefaciens* suspension and co-incubated for 10 minutes.

The cotyledons were then transferred, abaxial surface down, to solidified UM media supplemented with 3% (w/v) sucrose overlayed with one filter paper (8 per plate) and placed at 25°C for 2 days. The cotyledons were then transferred, axial surface up, to solidified MS media supplemented with 3% (w/v) sucrose, 0.04mgl^{-1} NAA, 0.5mgl^{-1} BAP, 100 µg/ml cefotaxime, 500 µg/ml carbenicillin and 50 µg/ml kanamycin and placed in a growth room at 25°C with a 16hr photoperiod. The explants were transferred to fresh medium every 14 days. After eight weeks, regenerating explants were transferred to solidified MS media supplemented with 0.11% (w/v) MES, 100 µg/ml cefotaxime and 50 µg/ml kanamycin.

Genomic DNA was isolated from shoots that had rooted and transgenic plants harbouring the constructs were selected following specific amplification of the CHR,CHI & IFS transgenes respectively.

Transgene positive plants were then transferred to 9cm diameter pots containing Levington M3 compost mixed with John Innes No.3 & perlite (3:3:2) and placed in a propagator in a growthroom at 25°C with a 16hr photoperiod. After 1 week the plants were removed from the propagator and maintained at 25°C with a 16hr photoperiod.

Leaf tissue harvested from each independent transformant and is stored at -80°C for subsequent flavonoid and isoflavonoid analyses as previously described.

Example 3: Transformation of potato

Stable transformation of *Solanum tuberosum L.* cv. Desiree

A.tumefaciens cells from transgene positive (PCR) colonies were used to inoculate a 20ml Lennox media broth containing kanamycin 50µg/ml and rifampicin 50 µg/ml and incubated for 3-days with shaking (120rpm) at 29°C. Following incubation, this culture was centrifuged at 3000*g* and the cell pellet resuspended in 25ml MS media (pH5.8) supplemented with 3% (w/v) sucrose.

Leaves were cut from 4-week old *Solanum tuberosum L.* plants, grown in tissue culture, and placed axial surface up onto solidified L3 medium [MS basal salts supplemented with 1.6% glucose, 0.8% agar, pH5.8] supplemented with 0.02mg/l NAA, 20mg/l GA_3, 2mg/l Zeatin riboside] and placed at 23°C for 2 days.

The excised leaves were then placed directly into the *A.tumefaciens* suspension and co-incubated for 10 minutes. Following co-incubation, the leaves were `blotted-dry' and transferred, axial surface up, to feeder plates (solidified L3 media overlayed with 2ml of tobacco cells suspension over which one filter paper was placed) and placed in darkness at 23°C for 2 days. The leaf explants were then transferred, axial surface up, to solidified L3 media supplemented 0.02mg/l NAA, 20mg/l GA_3, and 500 µg/ml cefotaxime and placed in a growth room at 23/C with a 16hr photoperiod for four days. The leaf explants were then transferred to fresh L3 medium supplemented with 0.02mg/l NAA, 20mg/l GA_3, and 500 µg/ml cefotaxime and 100mg/l kanamycin every 14 days. After approximately eight weeks, shoots (~1.5cm) were excised from the regenerating explants and transferred to solidified MS media supplemented with 1% (w/v) sucrose, 0.8% agar, 500 µg/ml cefotaxime and 100 µg/ml kanamycin.

Genomic DNA was isolated from shoots that had rooted and transgenic plants harbouring the constructs were selected following specific amplification of the CHR, CHI & IFS transgenes respectively.

Minitubers were initiated from each transgene positive plant by transfer of ~3cm long leaf node to MS media supplemented with 8%(w/v) sucrose and 0.8% agar and maintaining in darkness at 25°C. Minitubers were harvested from each independent transformant and stored at -80°C for subsequent flavonoid and isoflavonoid analyses.

Example 4:

Food product: Skin appearance benefits from isoflavone consumption

The investigation was designed as a double blind placebo controlled study with 33 female post-menopausal volunteers. The participants were randomised in a parallel design into two groups to receive foods with and without functional ingredients for a period of 12 weeks in total. For the duration of the study the subjects had to avoid soya containing foods and stop taking vitamins, minerals or other dietary supplements.

The study comprised two phases. Firstly, a "run-in" or "washout" phase when subjects consumed placebo foods for two weeks. Secondly, an intervention phase when subjects were randomly allocated to consume foods (2 low-calorie food bars per day) containing functional ingredients or placebo foods for a further 10 weeks.

Study foods were provided as a low-calorie bar, The bars were small (serving size 29g) and provided on average 108 calories and 3.1g fat.

Each functional bar contained:

Soya isoflavones	20mg
Green tea polyphenols	100mg
Gamma-linolenic acid	240mg
Carotenoids	0.25mg
Vitamin A	300µg
Vitamin C	60mg
Vitamin E	7.5mg
Vitamin B2	0.55mg
Vitamin B3	7mg
Vitamin B6	0.75mg
Vitamin D	5µg
Folate	200µg
Zinc	7.5mg
Calcium	600mg
PABA	120mg

The placebo foods contained the PABA (para-aminobenzoic acid) but none of the functional ingredients. PABA was added as a compliance marker to all the bars.

Consumption of the bars containing micronutrients of which the isoflavones are considered to most efficacious, resulted in a range of skin health and appearance benefits: i. Improved skin appearance and reduced signs of ageing due to reduced wrinkle height; ii. Improved firmness and skin tone; iii. Softer and smoother skin; iv. A less sensitive skin, that makes one feel better about their skin; v. Improved overall antioxidant status of the body and skin.

The statistical significance of each of the skin benefits or serum changes after ten weeks intervention is listed below:

Parameter	Week 10 'p' value
i. Wrinkle height (replicas)	< 0.078
ii. Firmness (indent value)	< 0.075
iii. Softness/Smoothness (Coefficient of restitution)	< 0.15
iv. Sensitive skin (questionnaire)	< 0.05
v. Serum antioxidant status (TEAC)	< 0.065